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Regulation of Milk Thistle (*Silybum marianum* L.) Growth, Seed Yield and Silymarin Content with Fertilization and Thidiazuron Application

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ABSTRACT

This study looks into the effect of foliar or soil fertilization and growth regulator thidiazuron (TDZ) treatment on the vegetative and reproductive growth, some physiological parameters, seed yield and silymarin content of field grown milk thistle (*Silybum marianum* L.) plants. Foliar fertilizer Agroleaf® of different NPK proportions was applied at different plant developmental stages. Combined application of the fertilizers with TDZ affected the growth, accumulation of nutrients (N, P, K), nitrate reductase activity, reducing sugars and free amino acids content positively. These changes were associated with altered flowering rate, enhanced seed ripening and increased yield. Treatment of milk thistle plants with TDZ in combination with foliar fertilizer increased seed yield due to an increase in the number of lateral stems, the number of flower heads and the seed fresh weight per flower head. Silymarin accumulation in the seeds was also positively influenced by the combined application of foliar fertilizer and TDZ.

Keywords: dry biomass, flowering dynamics, foliar and soil fertilization, lateral shoots, TDZ
Abbreviations: FF, foliar fertilization; **FF+TDZ**, foliar fertilization plus thidiazuron, **NRA**, nitrate reductase activity; **SF** soil fertilization; **SF+TDZ**, soil fertilization plus thidiazuron; **TDZ**, thidiazuron

INTRODUCTION

Milk thistle (*Silybum marianum* L., *Asteraceae* family) is a medicinal plant, cultivated for seed production. Seeds contain important substances used in the pharmaceutical industry. These substances, commonly known as silymarin, are powerful antioxidants (Dewick 1998). Silymarin primarily consists of an isomeric mixture of six phenolic compounds: silydianin, silychristin, diastereoisomers of silybin (silybin A and B), and diastereoisomers of isosilybin (isosilybin A and B) (Gus and Stermitz 2000; Lee *et al*. 2007). These compounds are flavonolignans, products of the phenyl-propanoid metabolic pathway in plants. Regulation of development in order to lengthen the reproductive stage length and to increase the yield of the seeds, with high silymarin content is an important problem for milk thistle cultivation. Enhanced milk thistle seed quantity and quality is dependent on the control of growth, flowering rate and transport of assimilates to seeds during the maturation process. Internal control of flowering includes perception of some external (day length, temperature) or internal (circadian rhythms, plant phase change, hormones) signals (Taiz and Zeiger 2002). The interaction between these factors is responsible for the synchronization of the plant reproductive process with environ-mental conditions. Among the external factors that can be successfully used for control of reproductive development of milk thistle are fertilization and growth regulators (Moor 1989). The control of flowering includes the action of some plant hormones such as gibberellins, cytokinins or auxins (Daphne *et al*. 2005).

Fertilization is a beneficial approach to regulate the reproductive development of plants. Foliar feeding with mineral nutrients has proved to be an effective regulator of nutritional disorders in plants. It would be conceivable to suggest that foliar fertilization cannot entirely replace the effect of soil fertilizers, but it may enhance the efficiency of soil nutrients assimilation (Eddy 2000; Wojcik 2004). The effects of foliar fertilization on the improvement of fruit quantity and quality have been reported by Eddy (2000) and Stancheva *et al*. (2004).

It could be expected that the appropriate fertilizer application and growth regulator can be effective tools to regulate milk thistle flowering rate, seed yield and quality. Among the group of plant growth regulators successfully used for control of flowering rate or seed ripening are cytokinins (Capelle *et al.* 1983). Thidiazuron (TDZ) (Dropp®) is a powerful plant growth regulator with high cytokinin activity, successfully used in tissue culture propagation or for regulation of plant growth or flowering (Cappelle *et al.* 1983; Alexieva *et al.* 1997).

The effect of foliar or soil fertilization and exogenously-applied TDZ during milk thistle cultivation on dry biomass, leaf content of the main macroelements, reducing sugars and amino acid accumulation, activity of leaf nitrate reductase, flowering dynamics, seed yield and silymarin accumulation are compared in this study.

MATERIALS AND METHODS

The study was conducted over three years (2004-2006) at an experimental field, on a leached cinnamonic meadow soil (Chromic Luvisols, according to FAO- Unesco-Isric legend). Milk thistle

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(*Silybum marianum* L.) seeds from cultivated plants (supplied from Sopharma AD, Bulgaria) were sown at a plant density of 5 plants m^{-2}. All treatments were arranged in randomized complete block design with four replications. The experimental plot area was 9 m^2. Mineral soil fertilizers (NH_4NO_3, triple super phosphate, K_2SO_4) applied to the soil before sowing in the following rates: N - 49.5 kg ha^{-1}, P_2O_5 - 138 kg ha^{-1} and K_2O - 150 kg ha^{-1}, were based on a soil test (the values of the initial NPK soil reserves). Three formulations of foliar fertilizers (Agroleaf®, Scotts Company, Ohio, USA) were applied at different developmental stages: (1) Agroleaf® total - N:P:K=20:20:20 + microelements, was applied twice during the vegetative growth stage on 20 days interval until the rosette phase; (2) Agroleaf® with high P - N:P:K=12:52:5 + microelements, was applied before the blooming stage; (3) Agroleaf® with high K - N:P:K=15:10:31 + microelements, was applied after the blooming stage. Microelements in chelated form are presented in concentrations: 0.1% Fe, 0.06% Mn, 0.06% Cu, 0.06% Zn, 0.02% B. Agroleaf® was applied by spraying under high pressure at rates of 5 kg ha^{-1} (0.5% solution, recommended by Scotts Company). Before spraying with Agroleaf®, a transparent plastic film covering each plot was used to avoid the penetration of dispersed foliar fertilizer into the soil. The film was removed after drying of the spray solution (approx. two hours after spatter).

The following treatments were tested: (1) control plants, without application of fertilizers and TDZ (C); (2) soil fertilization (SF); (3) soil fertilization plus TDZ (SF+TDZ); (4) foliar fertilization (FF); (5) foliar fertilization plus TDZ (FF+TDZ).

Thidiazuron (*N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea, TDZ, Dropp®, Schering Co.) with 50% active ingredient was applied as a 0.002% solution (8.33 g ha^{-1}) at the rosette stage of milk thistle development by spraying, ensuring thorough coverage of the plant.

Phenological observations were carried out to characterize the number of buds, flowers and mature flower heads containing ripe seeds, at five experimental time points: early flowering stage, flowering stage, immature flower heads, first stage maturity and mature flower heads. Total shoot dry biomass, shoot height, number of lateral stems per plant were measured at the flowering stage. Seeds yield at the final harvest were determined. Leaf total nitrogen was analyzed after Kjeldhal digestion on nitrogen analyzer Contiflo (Hungary). Leaf total phosphate was determined spectrophotometrically (Lowry and Lopez 1946; Skulatchov and Kiselev 1962). Total potassium was quantified by flame spectrometry. The activity of leaf nitrate reductase (NRA) was *in vivo* assayed by the method of Klepper *et al*. (1971). The leaf tissues (0.2 g) were placed in reaction mixture containing 4 ml 0.1 M potassium phosphate buffer (pH 7.5), 0.5 ml 0.1 M KNO_3, 0.5 ml 50% isopropanol and 0.05% chloramphenicol. The flasks were then wrapped in aluminum foil and were incubated in a water bath at 33°C with gentle shaking for 30 minutes. Nitrite formed was determined by measuring A_{540} after color development for 15 min with 1:1 mixture of 1% (w/v) sulphanilamide in 1.5 M HCl and 0.2% (w/v) *n*-naphthylethylenediamine dihydrochloride. For determination of the total free amino acids and soluble sugars dry leaves samples (0.025 g) were exhaustively extracted in boiling 25 ml 80% (v/v) ethanol. Ethanol soluble extracts were dried in a vacuum oven and soluble compounds were redissolved with 25 ml of distilled water. These water extracts were used for estimation of the total free amino acids and soluble sugars. Total free amino acids was determined according to Yemm and Cocking (1955). Citrate buffer (0.2 ml) – pH 0.5 and 1 ml ninhydrin reagent were added to 0.5 ml water extract. Fifteen minutes after water bath (80°C) incubation total free amino acids were measured spectrophotometrically at 570 nm. Soluble reducing sugars assay was done according to Dubois *et al.* (1956). One ml 5% phenol solution and 5 ml concentrated H_2SO_4 were added to the 0.5 ml of water solution. Thirty minutes after obtained complex compound was estimated spectrophotometrically at 490 nm.

Collected milk thistle seeds were dried at 50°C in a vacuum oven. Oil substances were extracted from 2.0 g dry seeds in 30 ml petroleum ether. The samples were evaporated in vacuum to dryness and dissolved in 95% methanol (Varma *et al.* 1980). The silymarin compounds (silydianin + silychristin and diastereoisomers of silybin + diastereoisomers of isosilybin) were analyzed by a Shimadzu LC-2010 HPLC apparatus equipped with a C18 column (125 mm, 5 µm, 4.0 mm), UV/VIS detector 288: elution flow rate was 1 ml min^{-1}, sample volume was 10 µl, solvent composition CH_3COOH : H_2O=70:30, isocratic regime.

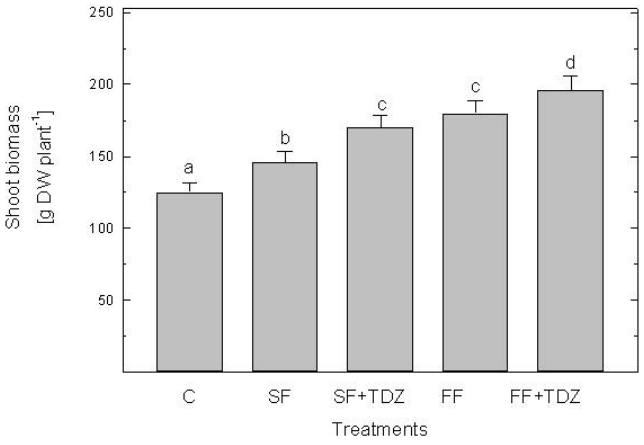

Fig. 1 Effect of soil and foliar applied fertilization on the dry aboveground biomass at the flowering stage of milk thistle plants treated with TDZ. Treatments: C - control plants, SF – soil fertilization, SF+TDZ - soil fertilization plus TDZ, FF – foliar fertilization, FF+TDZ - foliar fertilization plus TDZ. Bars indicate SE at three replication (n = 3).

Data from analysis are expressed as means of three-year experiments with four replications per year. Comparison of means was performed by the Fisher's LSD test (P = 0.05) after performing ANOVA analysis. The STASTICA (version 6.0) package was used for statistical analysis.

RESULTS

Soil (SF) and, especially, foliar fertilization (FF) resulted in a significant increase of aboveground dry biomass of milk thistle plants measured at flowering stage in comparison with the control (C) (**Fig. 1**). In variants with combined foliar feeding and TDZ treatment (FF+TDZ) was observed higher shoot biomass accumulation than the biomass values of milk thistle plants with soil fertilization and TDZ application (SF+TDZ).

Increased dry biomass accumulation can be considered a consequence of the increased lateral stem branching of plants after this kind of treatment (**Table 1**). Cytokinin activity of applied TDZ was manifested by an increase in the number of lateral shoots of milk thistle mainly after parallel treatments with foliar fertilizer. In both fertilized treatments with TDZ application, the number of lateral shoots increased compared to the control and to plants without TDZ.

The dynamics of flowering of cultivated milk thistle plants under conditions of different fertilization and TDZ treatments is represented in **Fig. 2**. Higher total number of flower heads, buds and flowers were observed in plants from SF and SF+TDZ treatments at early flowering stage. During the progress of the reproductive period the number of overblown flower heads increased especially in the fertilized plants treated with TDZ (SF+TDZ, FF+TDZ). At the milk thistle harvest (mature stage of milk thistle head deve-

Table 1 Effect of fertilization and TDZ treatment on plant height and lateral stem branching.

Treatments	Height of plant (cm)	№ of stems per plant
Control	52.6 ± 4.5 a*	2.6 ± 1.2 a
Soil fertilized plants	66.7 ± 3.3 b	4.0 ± 0.4 b
Soil fertilization+TDZ	78.6 ± 5.0 c	6.0 ± 0.6 c
Foliar fertilization	61.6 ± 3.4 b	5.0 ± 0.3 bc
Foliar fertilization+TDZ	76.4 ± 2.4 c	7.6 ± 0.2 d
LSD ($P \leq 0.05$)	10.30	0.81

* Data are presented as means from 3-year field experiment with four replications per year. Different letters indicate significant differences assessed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis.

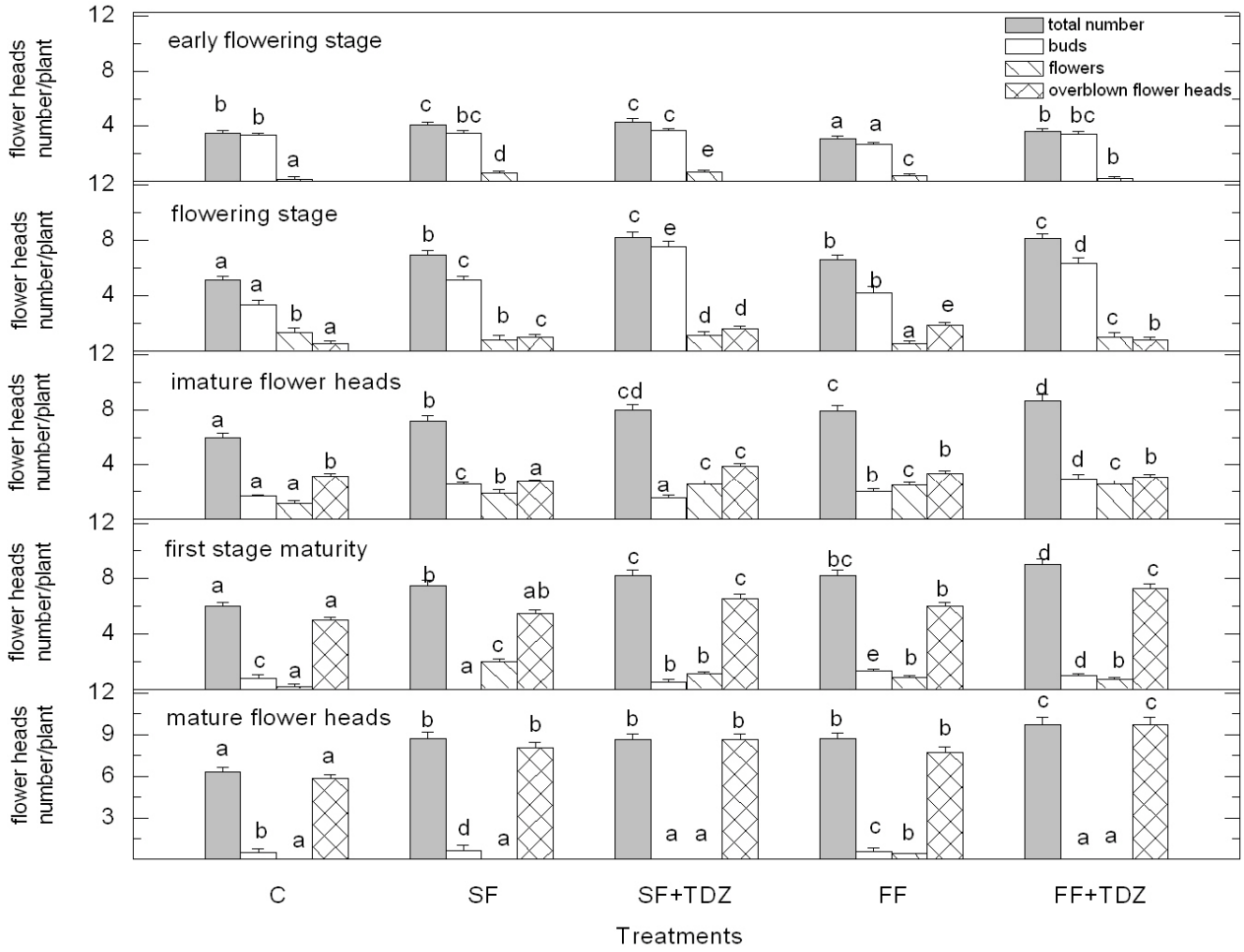

Fig. 2 Changes in the flowering dynamics of milk thistle plants grown at different fertilization and TDZ applications. Treatments: C, control plants; SF, soil fertilization; SF+TDZ, soil fertilization plus TDZ; FF, foliar fertilization; FF+TDZ, foliar fertilization plus TDZ. Bars indicate SE at three replication (n = 3).

lopment) foliar fed plants treated with TDZ showed the highest number of mature flower heads per plant compared to soil fertilized plants with TDZ and particularly to the control, where some buds were observed even at the harvest.

Combined treatment with foliar fertilizer and TDZ affected positively not only the appearance of additional reproductive organs but also influenced the rate of seed ripening. Thus, this treatment led to synchronization of the process of seed maturity, which can improve plant harvest efficiency.

The changes of shoot dry biomass were in correspondence to the changes of values of accumulated nitrogen and phosphorus in the leaves (**Table 2**). Leaf potassium values did not significantly differ among the treatments. During the flowering stage leaf nitrogen and phosphorus content significantly increased after the foliar feeding and TDZ treatment.

The increased assimilation of nitrogen in leaves was expressed with the activity of leaf nitrate reductase activity (NRA) (**Table 3**). Leaf NR activity showed maximum in the FF + TDZ treatments. The application of fertilization and TDZ addition stimulated NRA in comparison with the control. The activation of nitrogen assimilation in plants corresponded with the level of accumulated non-structural carbohydrates and free amino acids in plant leaves as well (**Table 3**). The highest values of these compounds were observed in plants treated with foliar fertilizer and TDZ.

Analysis of seed yield fertilized either through the soil or the leaves and treated with TDZ, showed increased number of flower heads per area unit (**Table 4**). Soil fertilization and foliar fertilization resulted in increased number of

Table 2 Accumulation of the main nutrients at flowering stage of plants in result of different fertilization and TDZ treatment.

Treatments	Leaves nutrient level (mg g^{-1} DW)		
	Nitrogen	Phosphorus	Potassium
Control	20.74 a*	2.38 a	16.52 a
Soil fertilization	20.37 a	3.76 b	20.95 b
Soil fertilization +TDZ	23.06 b	7.35 c	21.73 b
Foliar fertilization	21.55 ab	7.08 c	21.40 b
Foliar fertilization +TDZ	28.25 c	14.73 d	22.21 b
LSD ($P \leq 0.05$)	2.09	0.69	1.88

* Data are presented as means from 3-year field experiment with four replications per year. Different letters indicate significant differences assessed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis.

Table 3 Reducing sugars, total free amino acids and NR activity in the leaves of milk thistle plants at the early flowering stage in result of different fertilization and treatment with TDZ.

Treatments	Reducing sugars (mg g^{-1} FW)	Free amino acids (mg g^{-1} FW)	NR (nmol mg FW^{-1} h^{-1})
Control	2.64 a	13.10 a	204.73 a
Soil fertilization	2.86 a	16.80 b	246.00 b
Soil fertilization +TDZ	3.59 b*	19.86 c	293.00 c
Foliar fertilization	3.51 b	26.44 d	279.50 c
Foliar fertilization +TDZ	5.33 c	52.49 e	762.00 d
LSD ($P \leq 0.05$)	0.336	2.663	28.838

* Data are presented as means from 3-year field experiment with four replications per year. Different letters indicate significant differences assessed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis.

Table 4 Effect of fertilization and TDZ treatments on the structure of the seed yield of cultivated milk thistle plants.

Treatments	Number of flower heads per m^2 cultivated area	Seed fresh weight per flower head (g)	Seed yield ($g.m^{-2}$)
Control	31.70 a*	3.07 a	97.32 a
Soil fertilized	43.70 b	4.20 cd	183.54 c
Soil fertilized + TDZ	43.30 b	3.94 bc	170.60 bc
Foliar fed plant	43.55 b	3.66 b	159.39 b
Foliar fed+TDZ	48.75 c	4.32 d	210.60 d
LSD ($P \leq 0.05$)	3.87	0.352	15.15

* Data are presented as means from 3-year field experiment with four replications per year. Different letters indicate significant differences assessed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis.

flower heads, seed fresh weight and seed yield towards the control. Additional treatment with TDZ improved the yield process mainly in foliar fed plants. The foliar fed plants treated with TDZ showed the highest seed yield values – an increase with 116% compared to the control (C) and with 32% compared to the FF treatments. The most favorable effects on the seed yield of this combined treatment were a result of increased number of lateral stems, number of flower heads and greater seed fresh weight per flower head (**Tables 1, 4**).

Data derived from the chemical or HPLC analysis of seed total or individual compounds of silymarin showed no correspondence between seed yield and silymarin accumulation in most treatments (**Table 5**).

Seeds from the soil-fertilized plants contained less total silymarin compared to not fertilized control but fat substances were almost equal. Additional treatment with TDZ of the soil fertilized plants did not influence total seed yield, and total silymarin content. Foliar fertilization and thidiazuron treatment (FF+TDZ) ameliorated this negative dependence. Results indicate some increase of total silymarin in these seeds (10%) in comparison with of the control, but oil substances in the seeds remained unchanged. Concomitant increase of oil containing substances found in the seeds can contribute to the improved seed weight (**Table 4**).

DISCUSSION

The efficacy of the combined application of growth regulator and fertilizing procedure on plant growth improvement and nutrient-use efficiency of cultivated medicinal plants are rarely studied. Application of liquid fertilizers on plants usually results in fast accumulation of nutrients in leaves, although in some cases they can influence nutrient mobilization to roots as well (Wojcik 2004). The effect of treatments with fertilizers is explained mainly with the mechanism of improvement of assimilate partitioning, water or nutrients uptake and mobilization in plants, which can affect not only plant vegetation but reproductive growth as well (Omer and Ibrahim 1995; Omidbiagi and Nobakht 2001). This effect ensures regular development and improvement of flowering, seed development and ripening (Eddy 2000; Carrier *et al.* 2002). Liquid fertilizers can be more efficient in crops with low nutrient-use efficiency (Wojcik 2004). Such type of nutrient application can affect soil uptake rate of nutrients or can mobilize stored nutrients inside the plants. Moreover, liquid fertilizers are considered environment friendly and can control concentrations of nutrients in plants during particular stage of growth. The procedure of foliar fertilization is easy to combine with the application of some growth regulators, such as cytokinins, which are also known to control nutrient mobilization in plants (Moor 1989; Chernyadev 1994). The obtained results clearly demonstrate the advantage of combined foliar nutrition with different combinations of basic nutrients and TDZ application over soil fertilization. This approach was especially efficient with regard to improving flowering rate or seed ripening and maturity (**Fig. 2**). Genkov *et al*. (1997) found that exogenous application of cytokinins, including TDZ, improve stem branching, bud formation and flower setting or can overcome apical dominance in *Dianthus caryophyllus*. However, no data on its effect on milk thistle growth has been found. The biochemical mechanism of interactions between cytokinins and mineral nutrients inside the plants is basically unknown. One of the best-known effects of the action of cytokinins on nutrient accumulation is suggested to be through their regulation on cell proliferation and enlargement or through the delay of aging processes (Ferante *et al.* 2001). The effects of growth regulators on the phenol-flavonoid metabolism in plants are also largely unknown. Omidbaigi and Nobakht (Omidbiagi and Nobakht 2001) reported a negative correlation between the rate of nitrogen fertilization of field grown milk thistle plants and silymarin content in seeds. This is supported by our results for the treatments with soil fertilization (**Table 5**). When foliar fertilization was applied in combination with TDZ, negative dependence between seed yield quantity and quality obviously disappeared (**Tables 4, 5**). One of the reasons for this change may be the efficient role of nutrients in reproductive development (Eddy 2000). Stimulation of growth (**Fig. 1**) and nitrogen metabolism after combined treatment with liquid fertilizer and thidiazuron applications resulted in higher accumulation of non-structural carbohydrates and amino acids in the leaves which can enhance the process of flowering (**Table 3**). The accumulated soluble sugars or amino acids in leaves of plants were regarded as an internal signal for triggering the flowering process (Taiz and Zeiger 2002; Daphne *et al*. 2005).

On the other hand, some suggestions about the interrelations between nitrogen metabolism and phenol metabolism in phenol-propanoid metabolic pathway were proposed. Margna (1977) reported that higher accumulation of free amino acids in leaves can stimulate phenylalanine production and PAL activity which resulted in elevated phenolics production in the leaves and fruits of tomato plants. The release of ammonium following this reaction stimulates carbon metabolism to amino acid synthesis including phenylalanine. The late application of liquid fertilizer with higher potassium content can be regarded as a reason for the activation of carbohydrate metabolism as well (Kuepper 2003). Efficient mineral nutrition can also improve the leaf structure including vascular tissues thus ensuring more water and nutrients to reproductive organs. This process can accelerate the ripening of developing milk thistle seeds (Taiz and Zeiger 2002).

Table 5 Analysis of milk thistle seed silymarin content in result of different fertilization and TDZ treatment.

Treatments	Total silymarin in seeds (% dry weight)	Total lipid content (% dry weight)	HPLC analysis of individual silymarin compounds (% dry weight)	
			Silydianin + silychristin	Silybinin + iso-silybinin
Control	2.19 ± 0.7 b	13.37 ± 0.9 b	0.88 ± 0.03 c	1.31 ± 0.02 c
Soil fertilization	1.91 ± 0.6 a	14.01 ± 1.1 b	0.80 ± 0.01 b	1.11 ± 0.02 a
Soil fertilization+TDZ	1.94 ± 0.8 a	10.35 ± 0.3 a	0.78 ± 0.02 b	1.16 ± 0.03 b
Foliar fertilization	1.83 ± 0.4 a	9.64 ± 0.7 a	0.74 ± 0.03 a	1.09 ± 0.01 a
Foliar fed +TDZ	2.40 ± 0.2 c	14.29 ± 0.8 b	0.95 ± 0.01 d	1.45 ± 0.02 d
LSD($P \leq 0.05$)	0.190	1.480	0.040	0.038

* Data are presented as means from 3-year field experiment with four replications per year. Different letters indicate significant differences assessed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis.

CONCLUSIONS

Treatment with TDZ of foliar-fed plants was found to be an efficient way of changing not only the flowering dynamics but also of regulating growth, seed yield and quality. The accumulation of sugars and amino acids in leaves at flowering stage can be regarded as a precondition for flowering process synchronization. The changes of shoot dry biomass are in correspondence to the changes of accumulated nitrogen, phosphorus, reducing sugars and free amino acids in the leaves. Treatment of milk thistle plants with TDZ in combination with foliar fertilizer increased seed yield due to an increase in the number of lateral stems, number of flower heads and seed fresh weight per flower head. Foliar feeding combined with TDZ could be an effective device to ameliorate environmental effects and to overcome existing contradiction between seed yield quantity and quality during field cultivation of milk thistle.

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